

SCIENTIFIC REPORT

MMP inhibition prevents human lens epithelial cell migration and contraction of the lens capsule

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Purpose: The development of posterior capsule contraction following cataract surgery is caused by the activity of residual lens epithelial cells. Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes, which are essential for cell migration and cell mediated contraction following wound healing. The authors investigated whether inhibiting MMP activity can reduce lens epithelial cell migration and as a result, lead to a reduction in cell mediated capsule contraction.

Methods: Human donor lens capsules were cultured and treated with a broad spectrum MMP inhibitor, Ilomastat (GM6001). MMP-2 and MMP-9 production were determined by ELISA. Cell migration onto the posterior capsule and capsule contraction were digitally measured.

Results: MMP inhibition significantly reduced lens epithelial cell migration onto the posterior capsule ($p < 0.05$), and a reduction in capsule contraction was observed ($p < 0.05$).

Conclusions: Ilomastat significantly reduced lens epithelial cell migration onto the posterior capsule surface and inhibited capsule contraction. MMP inhibition may have a role in the therapeutic treatment of posterior capsule opacification.

Cataract is the most common pathology of the lens. It remains the leading cause of reversible blindness worldwide. Continual improvements in surgical technique and lens implant design have allowed the surgeon to improve on safety and visual outcome.^{1,2} Posterior capsule opacification (PCO) is the major cause of secondary visual loss following cataract surgery.³ A dramatic reduction in reported PCO rates from up to 50% following surgery down to as low as 7% have been due in part to advances in intraocular lens design over the last 20 years.^{4,5}

The proliferation and migration of residual lens epithelial cells (LECs) at the equator and under the anterior lens capsule following cataract surgery leads to the development of PCO.^{6–8} Repopulation of the posterior capsule by migrating LECs leading to capsule opacification with associated capsule wrinkling, contraction, and matrix production can result in significant visual impairment.

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes which are important in many physiological processes such as embryogenesis, development, and wound healing.⁹ Dysregulated MMP activity has long been implicated in diseases associated with uncontrolled proteolysis of connective tissue matrices such as arthritis,¹⁰ tumourgenesis,¹¹ and tissue ulceration.¹² MMPs have been found in normal ocular tissues¹³ with overexpression associated with excessive scarring.¹⁴ The use of synthetic MMP inhibitors in ocular disorders has been shown to reduce tissue damage.^{15,16} Our laboratory has recently shown that these agents inhibit a variety of cell mediated functions such as collagen synthesis, extracellular matrix contraction, and cell migration in Tenon's capsule fibroblasts in vitro.¹⁷

The aim of this study was to investigate the effect of the synthetic MMP inhibitor, Ilomastat (GM6001) on human lens epithelial cell migration and lens capsule contraction, in an in vitro model of PCO.

METHODS AND MATERIALS

Retrieval and preparation of human lens capsules

Donated human eyes were obtained from Moorfields Eye Bank (London, UK) and the Keratec Eye Bank (London, UK).

The research followed the tenets of the Declaration of Helsinki regarding the use of human material. The mean (standard deviation) ages of the donors were 72 (SD 8) years for the female donors and 69 years (SD 7) for the males. All lenses used in the study (total 16) were retrieved within 48 hours postmortem. Lens capsule bag culture was established using a technique previously described.¹⁸ Briefly, a sham cataract operation was performed, which involved anterior capsulorhexis, nuclear hydroexpression with phosphate buffered saline (PBS), and the aspiration of cortical lens fibres. The dissected lens capsule was transferred into a chamber of a 24 well culture plate. The lens capsule was cultured in 1 ml gelatinase free culture medium, which was composed of Dulbecco's modified Eagle's medium (DMEM) with penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), 2 mM L-glutamine, amphotericin (0.25 µg/ml), and 10% (vol/vol) fetal calf serum (FCS) (all Gibco Life Technologies, Paisley, UK). FCS was mixed with gelatin sepharose beads (Amersham Pharmaceutical) in a 1:9 ratio for 1 hour and then filtered to ensure that the serum was gelatinase free. The specimens were incubated at 37°C in 5% humidified CO₂ in air.

MMP inhibition

The effect of Ilomastat on cell migration and capsule contraction was investigated. The inhibitor was prepared as a 10 mM stock solution in dimethyl sulphoxide and test concentrations of 1 µM, 10 µM, and 100 µM were prepared by diluting with gelatinase free 10% serum containing culture medium. The negative control to Ilomastat (GM6001 negative control; Calbiochem) was also prepared and diluted to a concentration of 100 µM with culture medium. A pilot study had previously shown the presence of lens epithelial cells at the equator by day 3 of culture in 10% serum. Based on this observation, a study was designed and repeated four times, whereby the serum containing culture medium was replaced

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LECs, lens epithelial cells; MMP, matrix metalloproteinase; PBS, phosphate buffered saline; PCO, posterior capsule opacification.

with one of the four test media on day 3 and this marked the start of the study (day 0). The test media were changed daily.

MMP protein production

The culture media bathing the lens capsules were collected on days 5, 10, and 15; stored at -70°C then lyophilised and reconstituted in PBS before use. Total MMP-2 and MMP-9 were quantified in the collected culture media taken from the epithelial cell populated lens capsules by enzyme linked immunosorbent assay (ELISA). The ELISA kits were solid phase enzyme immunoassays based on a two step sandwich format and measured both the latent and active forms of the enzymes (Amersham BioSciences, UK).

A test run was performed for each assay to determine the appropriate dilution factor of 100 μl of each collected sample culture medium to ensure that the readings correlated with the exponential part of the respective standard curves. To eliminate the possibility of interassay variation for each ELISA, the samples were assayed in one run. The assays were performed in accordance with the manufacturer's instructions.

Lens epithelial cell migration

Epithelial cell migration onto the posterior capsule was measured by capturing a series of digital images taken on days 5, 10, and 15. The settings employed to photograph the capsules were standardised by using fixed camera and microscope settings and magnification respectively. The images were taken by attaching the camera lens to a customised microscope eyepiece. The distance travelled by the lens epithelial cells was measured from a predetermined mark drawn on the base of the culture dish at the leading edge of the epithelial cell population on the day the cells started to appear—that is, day 3 in culture, before the study started. The average distance travelled by the lens epithelial cells from the marked position was measured in pixels by using UTHSCSA (University of Texas Health Science Center in San Antonio) Imagetool Software. Measurements from the mark to the leading edge of the migrating cells were taken from 10 points on the mark. Each chosen point on the mark was a fixed distance apart, determined by measurements using the software.

Lens capsule contraction

Digital images of the lens capsules were captured and the measurements were again standardised as previously described. The vertical and horizontal capsule diameters between the pins were measured using UTHSCSA Imagetool Software, and the average diameter for each lens capsule was calculated from the total sum of the six repeated measurements at each axis.

Cell viability

To establish that the inhibitory effect of Ilomastat on cell migration was not caused by cell toxicity, cell viability was determined. Lens epithelial cell proliferation in the presence of 1 μM , 10 μM , and 100 μM Ilomastat was determined using a kit incorporating the WST-1 reagent (Boehringer Mannheim Diagnostics and Biochemicals, Lewes, UK) in accordance to the manufacturer's instructions. Briefly, lens capsules were cultured in serum until proliferating lens epithelial cells migrated and formed a confluent monolayer on the culture plate. The cells were then trypsinised and reseeded into 96 well tissue culture plates, at a density of 1×10^3 per well in serum containing culture medium and cultured for 4 hours. The culture medium was removed and the cells were washed three times with PBS before the addition of medium containing concentrations of Ilomastat (1 μM , 10 μM , 100 μM , and negative control). Three plates

per assay were seeded and prepared for harvesting on days 5, 10, and 15, the same time points used in the study. At each time interval 10 μl of WST-1 reagent was added to each well and incubated for 2 hours at 37°C . The absorbance, which relates to the number of viable cells converting the reagent to coloured formazan crystals, was read at 450 nm. The cells were re-fed daily with the test media.

Statistical evaluation

Statistical analysis was performed using Excel (Microsoft). Comparisons of data samples from contraction assays were performed by analysis of variance (ANOVA), which allowed multiple comparisons between two or more data sets. For all analyses, $p < 0.05$ was considered statistically significant with a 95% confidence interval.

RESULTS

MMP production during lens epithelial cell migration

Total MMP-2 and MMP-9 protein production increased with time with maximum levels reached on day 15. MMP levels were reduced with Ilomastat, with the greatest inhibitory effect being seen at the higher concentrations (10 μM and 100 μM , $p < 0.05$; fig 1). By day 10 a 50% reduction in total MMP-2 and MMP-9 levels was seen with 10 μM and 100 μM Ilomastat compared with control ($p < 0.05$).

Lens epithelial cell migration and capsule contraction

Epithelial cell migration onto the posterior capsule was significantly reduced by Ilomastat compared with control ($p < 0.05$; fig 2). On day 5, a twofold increase in the distance migrated was observed between control and Ilomastat treated capsules at the concentrations 10 μM and 100 μM . By day 10, a fivefold increase in the distance travelled was seen between control and 100 μM Ilomastat. In addition, increased capsule wrinkling in the control capsules limited accurate measurement of cell migration.

A confluent monolayer of lens epithelial cells was present in the control capsules at day 10 and evident capsule wrinkling and folds were seen (fig 3A and C). In contrast,

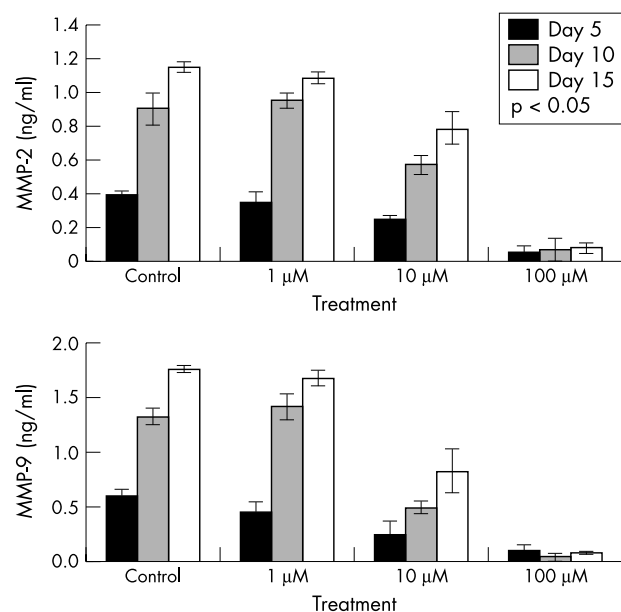


Figure 1 Total MMP-2 and MMP-9 protein production collected in the culture medium was quantified by ELISA. MMP levels were reduced by Ilomastat in a dose dependent manner compared with control (day 5, day 10, day 15; $p < 0.05$).

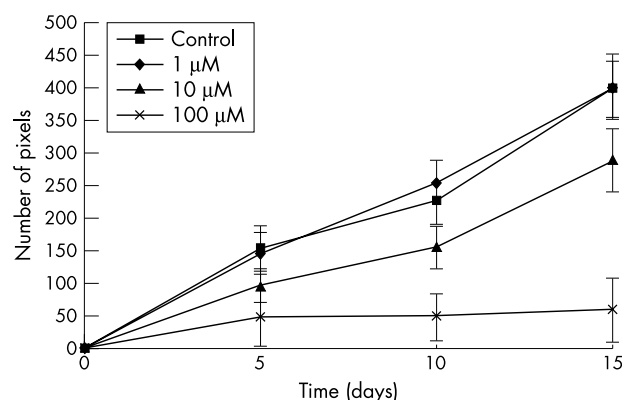


Figure 2 Serial digital photographs were taken of the lens capsules. The capsule diameters between the pins were measured using UTHSCSA (University of Texas Health Science Center in San Antonio) Imagetool Software, and the average diameter for each lens capsule was calculated from six repeated measurements from vertical and horizontal axes. All images were taken at the same marked area at $10\times$ magnification. Three capsules were measured at each treatment group. The graph illustrates the mean distance travelled by the cells and the error bars represent a 95% confidence interval.

minimal capsule wrinkling was noted with Ilomastat treatment (fig 3B and D). Finally capsule contraction, and thus the reduction of capsule diameter, was dose dependently inhibited by Ilomastat compared with control. Capsule diameter decreased by 60% in the control treated capsule compared to a 30% decrease with 10 μ M and less than 5% reduction with 100 μ M ($p < 0.05$, fig 4).

Cell viability

Lens epithelial cells remained viable and proliferated in the presence of all the concentrations of Ilomastat as detected by the WST-1 assay. No significant differences between control

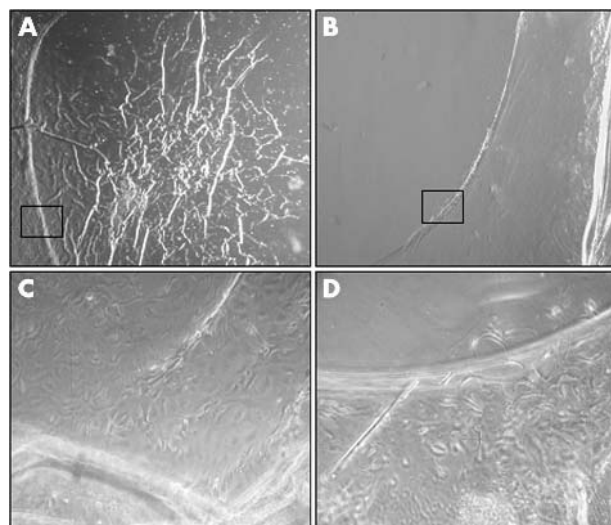


Figure 3 (A) Negative control treated lens capsule at day 15 demonstrating typical capsule wrinkling. The anterior capsule had also adhered onto the posterior capsule, which is not visible in the photograph. (B) Lens capsule that has been treated with 100 μ M Ilomastat at day 15. The capsule remains round with minimal capsule wrinkling compared with (A). At higher magnification of the boxed area of (A), the lens epithelial cells have clearly migrated over the posterior capsule to form a confluent layer (C). This contrasts with the marked area of (B), which shows virtually no lens epithelial cell migration onto the posterior capsule (D). (A and B, $\times 2.5$ magnification; C and D, $\times 10$ magnification.)

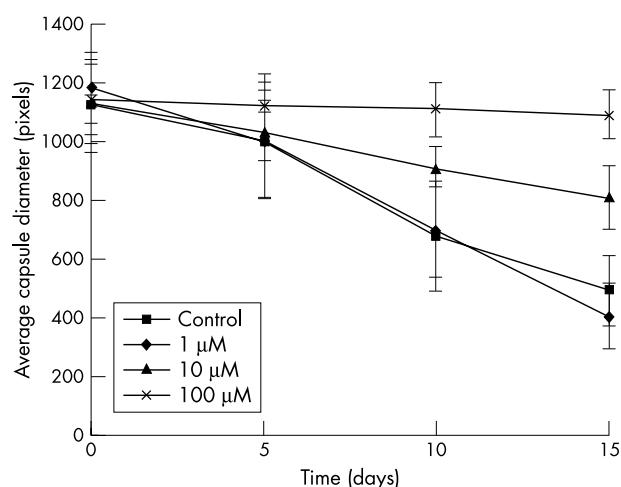


Figure 4 Cultured lens capsules were photographed at regular intervals and the average capsule diameters were digitally measured. The graph illustrates the effect of Ilomastat on capsule contraction. MMP inhibition resulted in a decrease in capsule contraction and therefore capsule diameter compared with control. Error bars indicating 95% confidence interval have been included but fall within the data points.

and Ilomastat (1 μ M, 10 μ M, and 100 μ M) treatments were found (fig 5).

DISCUSSION

Scar contracture plays an important role in the pathogenesis and failure of treatment of many ocular conditions, which include glaucoma, cataract (capsule contraction), trachoma, proliferative vitreoretinopathy, and diabetic retinopathy. Cell migration through the extracellular matrix along with collagen synthesis and deposition and matrix contraction are the key components to the scarring response. The data presented provide evidence to support the premise that MMP inhibition can reduce lens epithelial cell migration contraction of the capsule bag.

In response to surgery a wound healing response is triggered, and one of the key processes of healing is cell mediated contraction of the extracellular matrix. Residual lens epithelial cells are stimulated to proliferate and migrate onto the lens capsule by circulating growth factors and inflammatory cytokines.^{19, 20} Cell migration is intrinsically involved with matrix contraction and previous reports have shown a reduction in cell migration and matrix contraction by inhibiting MMP activity.^{17, 21} The use of MMP inhibitors to modulate the scarring response in other ocular structures has

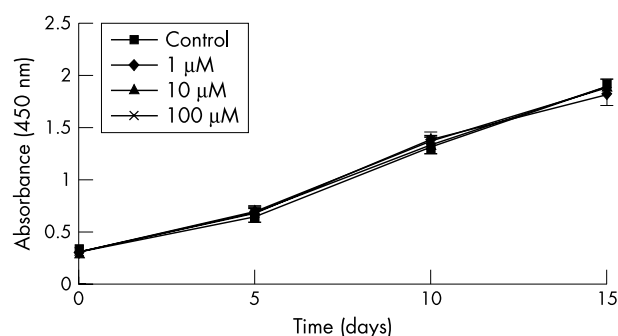


Figure 5 Viable LEC number measured with the WST assay. Cell proliferation in the presence of negative control, 1 μ M, 10 μ M, and 100 μ M Ilomastat was quantified. No significant difference was found between proliferation rates in the different treatment groups. Error bars represent standard error of the mean.

already been shown. Ozerdem *et al* have shown that progression of PVR indicated by scar tissue contraction can be significantly delayed with repeated intravitreal injections of the MMP inhibitor, prinomastat (AG3340).¹⁵ Furthermore, the level of MMP activity in the vitreous has been shown to be related to the development of retinal scarring.¹⁴ In addition, we recently reported a reduction in postoperative subconjunctival scarring associated with prolonged bleb survival following experimental glaucoma filtration surgery upon treatment with Ilomastat.²²

In this culture model of PCO, 10% serum was used to accelerate the cellular processes that would normally occur at a slower rate in the human eye following cataract surgery. We report a decrease in MMP protein production with Ilomastat treatment leading to a reduction in cell migration and capsular bag contraction. MMP-2 and MMP-9 production were identified. Other MMP members have also been reported in the lens and their possible role in the development of PCO has yet to be determined.^{13 23–25}

A link between fibroblast migration and wound contraction has been proposed by Harris, who demonstrated that fibroblasts seeded onto a thin film of heat polymerised silicone oil wrinkle the substrate when the cells attempt to migrate.²⁶ However, the cells do not move; the surface wrinkles. Harris proposed that the wrinkling was caused by cell tractional forces. This study provides further evidence of a possible link between migration and contraction as both process were inhibited to a similar degree by Ilomastat.

In this study we investigated whether MMP inhibition would reduce LEC migration. Cell migration onto the posterior capsule was dose dependently reduced with MMP inhibition. The magnification employed to record migration allowed clear identification of the LECs but limited measurement of the rate of cell locomotion to the first 7 days. A decrease in capsule contraction was observed alongside reduced cell migration with Ilomastat. By 7 days, prominent wrinkles and folds began to appear on the capsule. However, we found it difficult to accurately measure the actual distance travelled by the cells beyond this time due to capsule irregularity.

Several reports provide evidence that MMPs are involved in cell motility and contraction. Migrating fibroblasts are known to secrete collagenase,²⁷ and with the addition of tissue inhibitor of metalloproteinases, fibroblast mediated collagen gel contraction decreases.²⁸ Bullard *et al* reported impaired contraction in vitro by fibroblasts harvested from stromelysin-1 knockout mice compared with wild type.²⁹ In addition, Scott *et al* demonstrated a reduction in collagen gel contraction as a direct result of inhibiting cell migration. The authors found that these inhibitory effects were reversed upon removal of the MMP inhibitor, suggesting that exposure to the agent does not lead to cellular toxicity.²¹ The result from the cell proliferation assay we performed is in agreement with this observation, and has also been recently reported in our laboratory.¹⁷

The anterior chamber aqueous flare and cell reaction that develops from a breakdown in the blood-aqueous barrier has been reported to subside after one month of surgery.³⁰ Thus we hypothesise that MMP inhibition in the early stage following surgery may be all that is required to inhibit a significant amount of PCO development. Depending on the persistence of the MMP inhibitor in the lens bag, a slow release form may or may not be required. Possible slow release delivery systems are currently being investigated in our laboratory.

The molecular mechanisms that unite cell migration and wound contraction are not fully understood. As MMPs are capable of cleaving most matrix components, it is possible they play a role in integrin processing, modification of focal

attachment to the underlying matrix and cell locomotion. It remains to be determined which MMPs are responsible for cleavage of lens epithelial cell focal adhesion sites and whether inhibiting their activity reduces migration on the capsule.

Matrix contraction plays a role in the pathogenesis and failure of treatment of most visually disabling conditions. As such, our data suggest a key role for the modulation of MMP activity in the inhibition of matrix contraction. MMP inhibition resulted in a profound reduction in lens epithelial cell migration and subsequent capsule contraction. Our findings provide further evidence that MMP inhibition prevents cell migration and capsule bag contraction, potentially making it suitable for inhibiting PCO.

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ECHO.....

Isolation of a novel strain of adenovirus in epidemic keratoconjunctivitis



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A novel strain of adenovirus has been isolated from a 25 year old male patient in Japan suffering from severe clinical manifestations of epidemic keratoconjunctivitis (EKC). The novel causative agent, strain M86, showed similarities with two other adenoviruses: type 11 (Ad11) and type 35 (Ad35).

Although Ad11 infrequently causes keratoconjunctivitis, Ad35 and the new strain M86, have never before been reported as ocular pathogens. Analysis of M86 at a molecular level using restriction endonuclease showed that it might have evolved from the recombination of the two parent viruses Ad35 and Ad11.

The fibre knob of M86, used to attach the virus to a specific cellular receptor on conjunctival or corneal cells, was found to have 99% homology with Ad11. The hexon protein of M86, the antigen against which antibodies are raised to neutralise the infectivity of adenoviruses, was found to be partly homologous to Ad35 and partly to Ad11; the hypervariable regions (HVRs) of the hexon protein were 100% homologous to Ad35 in HVRs 1,2,3, and 6 and to Ad 11 in HVRs 4 and 6.

The mixed antigenic characteristics of a virus strain like M86 may enable it to circumvent existing immunity. This is important since it can enable the non-ocular adenoviruses to become ocular pathogens, which in the future might be responsible for outbreaks of EKC.

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